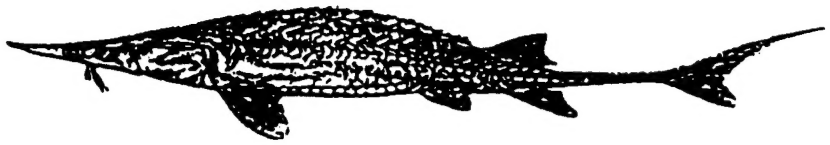
Genetic Studies of Scaphirynchus spp.

Pallid Sturgeon (Scaphirynchus albus)
Shovelnose Sturgeon (Scaphirynchus platorynchus)
Alabama Sturgeon (Scaphirynchus suttkusi)



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In fulfillment of Contract # DACW45-92-C-0064, as amended

for

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Planning Division
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Omaha, NE 68102-4978

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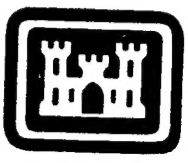

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13. ABSTRACT *(Maximum 200 words)*

The study was initially an analysis of morphologically identified pallid sturgeon, shovelnose sturgeon, and hybrid sturgeon using blood DNA. A total of 228 specimens were analyzed from different parts of the Mississippi River Basin. Prior to contract completion, another item was added to the contract to analyze one morphologically identified individual of Alabama sturgeon captured from the Alabama River. Since the specimen died without a blood sample being taken, a tissue sample was used to obtain DNA.

Contracted items were as follows:
1. Determine degree of genetic divergence between pallid and shovelnose sturgeon and describe taxonomic significance of this.
2. Identify genetically meaningful management units (stocks) within the range of the pallid for designing reintroduction efforts.
3. Determine if hybridization is occurring and whether it consists solely of F1 hybrids or if introgression is occurring.
4. Identify genetically meaningful management units (stocks) within the range of the shovelnose sturgeon.
5. Analyze tissue samples from one Alabama sturgeon, including developing the technique for extracting DNA material from tissue (instead of blood) samples, comparing this DNA with that already obtained from shovelnose and pallid sturgeon.

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sturgeon; pallid sturgeon; shovelnose sturgeon; hybrid sturgeon; Mississippi River Basin; hybridization

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FORWARD

The contracted study was initially an analysis of morphologically identified pallid sturgeon, shovelnose sturgeon, and hybrid sturgeon using blood DNA (contracted items 1-4). A total of 228 specimens were analyzed from different parts of the Mississippi River Basin. Speciments were identified in data tables by area of origin as follows:

DYR	Mo. River downstream from the Yellowstone River, Montana
DFP	Mo. River downstream from Fort Peck dam, Montana
UFP	Mo. River upstream from Fort Peck lake, Montana
DPOR	Yellowstone River downstream from the Powder River, Montana
DMR	Mississippi River downstream from the Missouri River
BPL	Blind Pony Fish Hatchery
DPR	Missouri River downstream of Platte River
DOR	Mississippi River downstream of Ohio River
ATR	Atchafalaya/Red River System
DO	Gavins Point National Fish Hatchery
WR	White River
DCD	Yellowstone River downstream of Cartersville Diversion
DWR	Mississippi River downstream of the Wisconsin River
DKR	Missouri River downstream of the Kansas River

Prior to contract completion, another item (item 5) was added to the contract to analyze one morphologically identified individual of Alabama sturgeon captured from the Alabama River. Since the specimen died without a blood sample being taken, a tissue sample was used to obtain DNA.

Contracted items within the scope of this study were as follows:

1. Determine degree of genetic divergence between pallid and shovelnose sturgeon and describe taxonomic significance of this.

2. Identify genetically meaningful management units (stocks) within the range of the pallid for designing reintroduction efforts.

3. Determine if hybridization is occuring and whether it consists solely of F1 hybrids or if introgression is occuring.

4. Identify genetically meaningful management units (stocks) within the range of the shovelnose sturgeon.

5. Analyze tissue samples from one Alabama sturgeon, including developing the technique for extracting DNA material from tissue (instead of blood) samples, comparing this DNA with that already obtained from shovelnose and pallid sturgeon.

The homogeneity of allele frequencies at the prealbumin-related sequence locus among pallid sturgeon and among shovelnose sturgeon suggested that sufficient gene flow exists to prevent formation of genetically meaningful management units (stocks). Documentation of allelic variability at only one locus precluded any assessment of hybridzation as well. Detailed (item by item) analysis of objectives 1 - 4 by the contractor can be found in Appendix 1. The section of the report on the Alabama sturgeon answers objective 5.

Interim reports from the contractor referenced in the final analysis can be found in Appendix 2. These early reports contain details on methodology.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other documents. The views and opinions of the authors of this report do not constitute an official position of the U.S. Fish and Wildlife Service.

Additional copies of this report may be obtained from the Omaha District Army Corps of Engineers at the address listed on the cover.

This report should be referenced as follows:

Genetic Analyses Inc. 1994. Genetic Studies of Scaphirynchus spp. Unpublished report for the U.S. Army Corps of Engineers, Omaha District; U.S. Fish and Wildife Service, Bismarck, North Dakota; U.S. Army Corps of Engineers, Mobile District.

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STURGEON GENETIC STUDIES: THE CURRENT "BOTTOM LINE"

An Interpretation For Layfolk

There have been three genetic studies conducted to date assessing relationships of sturgeons of the genus *Scaphirhynchus*, the pallid, shovelnose, and Alabama sturgeons. All three have attempted to discover consistent genetic differences between pallid and shovelnose sturgeons, and two of the three have compared all three sturgeons. Two of the studies have examined variation in nuclear DNA, one directly (the Genetic Analyses study of PCR-amplified DNA fragments) and one indirectly (the allozyme study of 37 gene products of Phelps and Allendorf (Copeia 1983: 696-700)). The third study, supervised by Schill, compared sequences of segments of the mitochondrial DNA cytochrome b gene. The results can be reviewed from the perspective of two major questions: 1) are pallid and shovelnose sturgeons different species, and if so, what is the extent of hybridization between them; and 2) is the Alabama sturgeon genetically distinct from either pallid or shovelnose sturgeons?

Pallid versus Shovelnose: None of the studies detected significant genetic differences between pallid and shovelnose sturgeons. The mtDNA sequences of one individual of each species were identical, and the four polymorphic loci identified in the other two studies yielded similar allele frequencies for each species. All other loci and fragments examined were very similar or identical. These results would be expected if pallid and shovelnose sturgeons were in fact the same species. Does this guarantee that they are one species? No. If the two types had diverged very recently or if *Scaphirhynchus* molecular evolution were very slow, sequences and allele frequencies may have diverged only a little from the ancestral type. Alternatively, there may be sufficient gene exchange (i.e., hybridization) to bring allele frequencies to equilibrium frequencies; Slatkin has shown that only a few exchanges/generation are enough to produce genetic identity. In sum, there is no genetic evidence supporting the contention that pallid and shovelnose sturgeons are different species, but there is strong but insufficient evidence to conclude that they certainly are one species. How much evidence for conspecificity is enough? This question probably cannot be answered, but if no genetic differentiation can be demonstrated after repeated efforts, the only genetic consideration in recovery efforts is avoidance of loss of genetic variability in each management unit established by morphological criteria.

Alabama Sturgeon: The two genetic studies conducted so far have reached different conclusions. The mtDNA sequence of the single Alabama individual is identical to that of both pallid and shovelnose sturgeons. At the nuclear DNA level, the Alabama individual has the common sturgeon genotype at the one polymorphic locus analyzed, but when the total available nuclear DNA markers are assessed, considerable genetic differences between the Alabama and both shovelnose and pallid sturgeon individuals are observed. Is the Alabama sturgeon the same species as shovelnose, pallid, or both? The nuclear DNA differences suggest that the Alabama sturgeon is much more different genetically from either shovelnose or pallid than the latter two species are from each other. However, this result is based upon only one individual. A second individual would document whether the observed differences are consistent; based upon the very low levels of geographic and individual variability observed in pallid and shovelnose sturgeons, one more individual should be enough to test consistency. Genetic differentiation without question implies the presence of two separate gene pools, whether or not they are described as species.

PALLID, SHOVELNOSE, HYBRID STURGEON STUDIES
FINAL REPORT 10/94
GENETIC ANALYSES, INC.

The purpose of this report is to present final results of a genetic study of pallid and shovelnose sturgeons, discuss conclusions which can be drawn from the final data set, and recommend directions of future research to address as yet unresolved issues critical in designing recovery plans. The results presented here are completed analyses of preliminary studies described in the 1/94 Interim Report, which is attached to provide methodological detail. The implications of the genetic data will be discussed pursuant to two critical questions: 1) do genetic markers exist which are reliably diagnostic to identify sturgeon individuals as pure pallid, pure shovelnose, or interspecific hybrids?; and 2) do genetic data support the current systematic decision that pallid and shovelnose sturgeons are distinct species of the genus *Scaphirhynchus*?

Overview of genetic methodologies

Eight DNA primers were designed from conserved regions of nuclear genes which consistently amplified sturgeon genomic DNA fragments. Preliminary screens of a large number of restriction enzyme digests identified a prealbumin amplimer polymorphism in shovelnose and pallid sturgeon DNA digested with Sau96I, yielding by polymerase chain reaction (PCR) amplification four fragments, which upon gel purification appeared to represent products of variant alleles at a single locus. This prealbumin polymorphism was selected for intensive analysis in all shovelnose and pallid sturgeon samples available, representing some 228 individuals. Analyses of allele frequencies at this prealbumin-related locus will be presented below.

In a second genetic study, an attempt was made to compare levels of genetic divergence in the genus *Scaphirhynchus* (i.e., shovelnose vs. pallid) with those in another sturgeon genus *Acipenser* (lake sturgeon, Atlantic sturgeon, and Gulf sturgeon). An admittedly rough estimate of genetic distance was made using all eight primer pairs and estimating the number of PCR amplification fragments common to each taxon pair. This estimate is summarized as the number of shared fragments divided by the total number of different fragments resolved. Results of these analyses will be discussed below regarding certainty of specific status of the pallid sturgeon.

Details of primer construction, polymerase chain reaction (PCR) amplification, and DNA sample preparation can be found in the attached 7/93 and 1/94 interim reports. [Sequences for these primers have been provided in preliminary reports, but because they were custom-designed for other studies and have not yet been published, these sequences should be considered proprietary at present.] Tables 1 and 2 list sturgeon DNA samples analyzed and general locality data. It should be emphasized that collection of tissue samples for DNA extraction in these studies was performed by many individuals in a variety of field conditions, producing

unavoidable variability in DNA quality. A qualitative estimate of relative DNA quality is included for each sample in Table 1 as a guideline for suitability for future use in genetic studies.

I. Genetic variability at the prealbumin-related locus in shovelnose and pallid sturgeons

The 1.7 kilobase (kb) fragment produced by PCR amplification of sturgeon genomic DNA using prealbumin forward and reverse primers yielded polymorphic restriction fragments upon digestion with Sau96I, resulting in three fragment phenotypes: 1) 985 and 715 base pair (bp) fragments, designated as a presumptive *AA* genotype; 2) 375, 610, and 715 bp fragments, designated the *BB* genotype; and 375, 610, 715, and 985 bp fragments, considered to be a heterozygous *AB* genotype. The molecular polymorphism, then, appears to result from the presence or absence of a Sau96I cleavage site in the 985 bp fragment. Geographic distributions of these genotypes are summarized in Table 2. Two salient features of the results are apparent. First, each population sample and each morphologically determined species sample, as well as the total data set summed over species (4 *AA*, 61 *AB*, 141 *BB*, N=206), has genotypic proportions in close agreement with Hardy-Weinberg expectations; the evidence is thus quite strong that the observed genotypes result from two alleles at a single prealbumin-related locus. Second, the difference in allele frequencies between morphologically determined shovelnose sturgeons (A = 0.1832, N=131) and pallid sturgeons (A = 0.098, N=46), while moderately large, is statistically insignificant ($z = 1.55$, $P > 0.05$). Assuming that pallid and shovelnose sturgeons are distinct species, the argument could be made that representation of the *A* allele in pallid sturgeons is due to inclusion of pallid-like hybrids in the pallid sample, and that the *A* allele actually is found only in shovelnose sturgeons. The absence of *A* alleles in sampled pallid sturgeons from the downstream Yellowstone River, considered to exhibit no evidence of potential shovelnose introgression, enhances the plausibility of such an hypothesis. The result from lake sturgeons that only the apparent *B* allele is detected certainly suggests that the *B* allele is the plesiomorphic character state at the locus. Even if the *A* allele were truly absent in pallid sturgeons (which could be suggested if the 9 heterozygous individuals evidenced potential hybrid origin upon reexamination), the diagnostic value of the locus is limited in that more than 65% of shovelnose sturgeon individuals would be expected to possess the *BB* genotype identical to pallids. However, if pallid sturgeon management programs are undertaken, it seems reasonable to minimize frequency of prealbumin *A* alleles, or to maintain their frequency at no greater than 10% in the managed population. Such a measure would assuredly avoid at least a small amount of shovelnose introgression.

It should be emphasized that the prealbumin-like locus was selected for genotypic analysis because it exhibited the clearest evidence for potentially "species-specific" polymorphism in preliminary analyses. As an indication of how infrequent such polymorphisms are in the present sample, only Sau96I digests produced polymorphic fragments among 20 restriction enzymes which cut prealbumin-related sturgeon DNA (see Table 1 in appended 1/94 interim report). These results

raise serious questions regarding the specific status of pallid sturgeons, which were addressed further by a survey of all available, amplifiable primers.

II. Genetic variability of PCR-amplified fragments in *Scaphirhynchus* and *Acipenser* sturgeons

The lack of discrete morphological characters for diagnosis of pallid and shovelnose sturgeons has been a severe limitation in estimating frequency of hybridization between the two presumptive species. The very high DNA sequence similarity between presumed pallid and shovelnose individuals at the 1.7 kb prealbumin-related locus and the lack of significant allelic frequency differences between the two forms raises further doubts of the validity of the pallid sturgeon as a distinct *Scaphirhynchus* species. Unfortunately, no adequate surveys of genetic variability at large numbers of loci have been undertaken in *Scaphirhynchus* sturgeons. (The only survey published to date, by Phelps and Allendorf in (1983) failed to identify any diagnostic alleles or even significantly different allelic frequencies at 37 isozyme loci in sizeable samples of shovelnose and pallid sturgeon populations). As a crude estimate of genetic distance, the five primer pairs yielding reproducible PCR amplification were used to estimate pairwise fragment sharing between *Scaphirhynchus* and *Acipenser* species samples. The results presented in Table 3 are in sharp contradistinction of equal levels of divergence between *Acipenser* and *Scaphirhynchus* taxa. From a total of >50 identifiable fragments, the proportion of bands shared between *Acipenser* species is considerably higher than with either shovelnose or pallid samples, as would be expected, but the proportion of bands shared by *A. fulvescens* and *A. oxyrhynchus* (3 localities, two nominal subspecies) is vastly lower than that shared by shovelnose and pallid sturgeon samples from the Missouri river system. The proportion of bands shared by *A. oxyrhynchus* from different localities is in fact considerably lower than that of the *Scaphirhynchus* individuals. The *Acipenser* data document that substantial genetic differentiation has occurred during sturgeon evolution in an least some taxa; all the available genetic data, on the other hand, indicate little or no differentiation within *Scaphirhynchus*. Of course, it is possible that one of the *Scaphirhynchus* individuals used in our second study was misidentified and in fact a conspecific comparison has been made; however, this merely would illustrate further inadequacy of morphological identification. The other, quite strong possibility remains that pallid and shovelnose sturgeons are actually morphs distinguished primarily by size of a single *Scaphirhynchus* species. Should that hypothesis be true, attempts to identify diagnostic genetic markers may be futile.

Conclusions and recommendations for future research

As listed in Table 1, the prealbumin-like restriction fragment polymorphism in *Scaphirhynchus* has been highly reproducible in repeated digestions and amplifications of DNA samples. All the results obtained in the present study suggest conspecificity of pallid and shovelnose sturgeons. This appears to be the hypothesis in urgent need of intensive testing. Several recommendations for future research thus can be made:

1) Reexamine all sturgeons heterozygous for prealbumin-like alleles and identified by morphological characters as pallid sturgeons, for evidence of possible hybrid origin.

2) Increase sample sizes of pallid sturgeons to determine whether prealbumin-like *A* allele frequencies are significantly lower than in shovelnose sturgeon samples.

3) Increase sample sizes of pallid and shovelnose sturgeons for band-sharing comparisons of amplification fragments of multiple PCR primers.

4) Assemble an independent data set of allele frequencies at ≥ 50 isozyme loci in pallid and shovelnose sturgeons, and ideally, including comparisons of lake and Atlantic sturgeons.

5) Assemble a central collection of isozyme and DNA samples which can be accessed by multiple investigators to assure cross-comparability of results of future genetic studies.

The question of whether the pallid sturgeon is a valid species obviously is critical, given the level of investment in sturgeon management. The data sets produced during the research described here certainly raise the validity question to a new level of urgency, but more adequate sampling and comprehensive genetic analyses are necessary for a definitive answer to the question. It is important to realize that disproving the hypothesis that pallid and shovelnose sturgeons are different species is extremely difficult; until the weight of evidence for genetic identity becomes overwhelming (e.g., similar allele frequencies at a larger number of polymorphic loci, occurrence of the same rare alleles in both taxa, identity of DNA sequences at a larger number of nuclear and mitochondrial DNA segments), the post facto arguments that researchers simply have looked at the wrong genes or that hybridization has obscured genetic differences can always be advanced. The current situation is similar to that which has continued for decades in resolution of the status of the red wolf, with strong opinions prevailing on both sides of the species issue, to the detriment of preservation programs and to support of research and management efforts under the Endangered Species Act. It is vital to continued progress in preservation of biodiversity and ecosystems that taxonomic debates be minimized, and that the best genetic differentiation data possible be provided for design of achievable recovery plans.

Table 1. List of samples, data analyses, genotypes, and DNA quality estimates

Specimen	Designation	# of Amplifi-cations	# of Sau 96I Digests	Genotype	Estimate of DNA Quality*
ST 1	N/A	N/A	N/A	N/A	N/A
ST 2	DYR-S1	5	1	BB	F
ST 3	DYR-S2	3	1	BB	F
ST 4	DYR-S3	5	1	BB	F
ST 5	DYR-S4	3	1	BB	F
ST 6	DYR-S5	3	1	BB	F
ST 7	DYR-S6	3	1	AB	F
ST 8	DYR-S7	6	1	BB	F
ST 9	DYR-S8	3	1	BB	F
ST 10	DYR-S9	4	2	AB	F
ST 11	DYR-S10	3	1	BB	F
ST 12	DYR-S11	2	1	BB	G
ST 13	DYR-S12	2	1	AB	G
ST 14	DYR-S13	2	1	BB	G
ST 15	DYR-S14	2	1	BB	G
ST 16	DYR-S15	3	1	BB	G
ST 17	DFP-S1	3	1	BB	G
ST 18	DFP-S2	4	1	BB	G
ST 19	DFP-S3	2	1	BB	F
ST 20	DFP-S4	2	1	AB	F
ST 21	DFP-S5	3	1	BB	F
ST 22	DFP-S6	3	1	BB	F
ST 23	DFP-S7	3	1	AB	F
ST 24	DFP-S8	2	1	BB	G
ST 25	DFP-S9	2	1	AB	G

* E=Excellent; G=Good; F=Fair; P=Poor

Specimen	Designation	# of Amplifi-cations	# of Sau Digests	Genotype	Estimate of DNA Quality
ST 26	DFP-S10	2	1	BB	G
ST 27	DYR-S16	3	1	BB	F
ST 28	DYR-S17	3	1	BB	F
ST 29	DYR-S18	6	1	BB	P
ST 30	DYR-S19	5	1	BB	F
ST 31	DYR-S20	4	1	BB	F
ST 32	UFP-P1	2	1	AB	E
ST 33	UFP-P2	2	1	AB	E
ST 34	UFP-P3	3	0		P
ST 35	UFP-P4	2	1	BB	E
ST 36	DFP-S11	2	1	BB	E
ST 37	DFP-S12	2	1	AB	E
ST 38	DFP-S13	2	1	BB	E
ST 39	DFP-S14	2	1	AB	E
ST 40	DFP-S15	2	1	BB	E
ST 41	DFP-S16	2	1	BB	E
ST 42	DFP-S17	2	1	BB	E
ST 43	UFP-S1	2	1	AB	E
ST 44	UFP-S2	2	1	BB	G
ST 45	UFP-S3	2	1	BB	F
ST 46	UFP-S4	2	1	BB	F
ST 47	UFP-S5	2	1	BB	F
ST 48	UFP-S6	2	1	BB	F
ST 49	UFP-S7	2	1	BB	F
ST 50	UFP-S8	2	1	BB	F

Specimen	Designation	# of Amplifi-cations	# of Sau Digests	Genotype	Estimate of DNA Quality
ST 51	UFP-S9	7	2	BB	P
ST 52	UFP-S10	3	1	BB	F
ST 53	UFP-S11	6	1	BB	F
ST 54	UFP-S12	6	1	AB	F
ST 55	UFP-S13	6	2	AB	F
ST 56	UFP-S14	2	1	BB	G
ST 57	UFP-S15	4	3	A ?	F
ST 58	UFP-S16	2	1	BB	E
ST 59	UFP-S17	2	1	BB	E
ST 60	UFP-S18	2	1	BB	E
ST 61	UFP-S19	6	1	BB	G
ST 62	UFP-S20	2	1	BB	F
ST 63	DPOR-S1	4	1	BB	F
ST 64	DPOR-S2	6	1	BB	G
ST 65	DPOR-S3	11	1	AB	P
ST 66	DPOR-S4	4	2	BB	F
ST 67	DPOR-S5	3	1	BB	F
ST 68	DPOR-S6	4	2	BB	F
ST 69	DPOR-S7	3	2	AB	G
ST 70	DPOR-S8	3	2	BB	G
ST 71	DPOR-S9	4	1	BB	F
ST 72	DPOR-S10	5	1	BB	F
ST 73	DPOR-S11	4	2	BB	F
ST 74	DYR-P1	3	1	BB	G
ST 75	UFP-P5	3	2	BB	F

Specimen	Designation	# of Amplifi-cations	# of Sau Digests	Genotype	Estimate of DNA Quality
ST 76	UFP-P6	3	1	BB	F
ST 77	DMR-H1	12	3	AB	P
ST 78	DMR-H2	10	2	BB	P
ST 79	DMR-H3	9	2	AB?	P
ST 80	DMR-H4	5	1	AB	F
ST 81	DMR-H5	4	1	AB	F
ST 82	DMR-H6	5	1	BB	F
ST 83	DMR-H7	5	1	AB	F
ST 84	DMR-H8	5	1	BB	F
ST 85	DMR-H9	8	1	BB	F
ST 86	DMR-H10	5	1	BB	F
ST 87	DMR-H11	4	1	BB	F
ST 88	DMR-H12	4	1	BB	F
ST 89	DMR-H13	2	1	BB	P
ST 90	DMR-H14	3	1	BB	G
ST 91	DMR-H15	3	1	AB	F
ST 92	DMR-H16	5	1	BB	F
ST 93	DMR-H17	3	1	BB	F
ST 94	DMR-H18	3	2	BB	F
ST 95	DMR-H19	12	4	AA	P
ST 96	DMR-H20	3	1	BB	F
ST 97	BPL-1	4	1	BB	G
ST 98	BPL-2	4	1	BB	G
ST 99	BPL-3	4	1	BB	G
ST 100	BPL-4	4	1	BB	G

Specimen	Designation	# of Amplifi-cations	# of Sau Digests	Genotype	Estimate of DNA Quality
ST 101	BPL-5	4	2	BB	G
ST 102	DPR-P1	10	1	BB	P
ST 103	DPR-P2	3	1	BB	F
ST 104	DOR-P1	3	1	BB	F
ST 105	DOR-P2	4	1	AB	F
ST 106	DMR-P1a	3	1	AB	F
ST 107	DMR-P1b	3	1	BB	F
ST 108	DMR-P2	10	1	AB	P
ST 109	DYR-P2	3	1	BB	G
ST 110	DYR-P3	2	1	BB	F
ST 111	DYR-P4	11	1	BB	P
ST 112	DYR-P5	3	1	BB	F
ST 113	DYR-P6	2	1	BB	E
ST 114	DYR-P7	2	1	BB	E
ST 115	DYR-P8	8	1	BB	P
ST 116	DYR-P9	9	1	BB	P
ST 117	DYR-P10	7	1	BB	F
ST 118	UFP-P7	2	1	BB	F
ST 119	ATR-S1	2	1	BB	F
ST 120	ATR-S2	2	1	AA	F
ST 121	ATR-S3	2	1	BB	F
ST 122	ATR-S4	2	1	AA	G
ST 123	ATR-S5	2	1	BB	G
ST 124	ATR-S6	2	1	BB	G
ST 125	ATR-S7	2	1	BB	G

Specimen	Designation	# of Amplifi-cations	# of Sau Digests	Genotype	Estimate of DNA Quality
ST 126	ATR-S8	2	1	BB	G
ST 127	ATR-S9	2	1	AB	F
ST 128	ATR-S10	2	1	AB	F
ST 129	ATR-S11	9	1	AB	P
ST 130	ATR-S12	9	1	BB	P
ST 131	ATR-S13	2	1	BB	F
ST 132	ATR-S14	2	1	BB	F
ST 133	ATR-S15	2	1	BB	F
ST 134	ATR-S16	2	1	AB	E
ST 135	ATR-S17	2	1	BB	E
ST 136	ATR-S18	4	2	AB	E
ST 137	ATR-S19	2	1	BB	G
ST 138	ATR-S20	2	1	AB	E
ST 139	ATR-P1	2	1	AB	E
ST 140	ATR-P2	2	1	BB	E
ST 141	ATR-P3	2	1	BB	E
ST 142	ATR-P4	2	1	BB	E
ST 143	ATR-P5	2	1	AB	E
ST 144	ATR-P6	2	1	BB	E
ST 145	ATR-P7	2	1	BB	E
ST 146	ATR-P8	2	1	BB	F
ST 147	ATR-P9	2	1	AB	F
ST 148	ATR-P10	11	3	AB	P
ST 149	ATR-H1	8	2	AB	P
ST 150	ATR-H2	2	1	AB	G

Specimen	Designation	# of Amplifi-cations	# of Sau Digests	Genotype	Estimate of DNA Quality
151	ATR-H3	2	1	AB	G
152	ATR-H4	2	1	AB	E
153	ATR-H5	2	1	BB	E
154	ATR-H6	2	1	BB	E
155	ATR-H7	2	1	BB	E
156	ATR-H8	2	1	AB	E
157	ATR-H9	2	1	BB	E
158	ATR-H10	2	1	BB	E
159	UFP-P8	2	1	BB	E
160	UFP-P9	2	1	BB	G
161	DO-P1	2	1	BB	E
162	DO-P2	2	1	BB	E
163	DO-P3	2	1	BB	G
164	DO-P6	2	1	BB	G
165	DO-P8	2	1	BB	G
166	DYR-P1	2	1	BB	E
167	DFR-P1	2	1	BB	E
168	UFP-P10	2	1	BB	E
169	DFP-P1	2	1	BB	E
170	DYR-P11	6	1	BB	P
171	WR-S1	2	1	AB	G
172	WR-S2	2	1	AB	E
173	WR-S3	2	1	AB	F
174	WR-S4	2	1	AB	F
175	WR-S5	2	1	AB	G

Specimen	Designation	# of Amplifi-cations	# of Sau Digests	Genotype	Estimate of DNA Quality
176	WR-S6	2	1	BB	F
177	WR-S7	2	1	BB	F
178	WR-S8	2	1	AB	F
179	WR-S9	2	1	AB	F
180	WR-S10	2	1	AB	F
181	WR-S11	11	1		P
182	WR-S12	2	1	AB	F
183	WR-S13	2	1	AB	F
184	WR-S14	2	1	BB	G
185	YR-P1	2	1	BB	E
186	YR-P2	2	1	BB	E
187	DFP-S18	2	1	BB	G
188	DFP-S19	2	1	BB	E
189	DFP-S20	2	1	BB	E
190	UFP-P11	2	1	BB	E
191	DCD-S1	3	1	AB	E
192	DWR-S1	2	1	AB	E
193	DWR-S2	2	1	AB	E
194	DWR-S3	3	1	BB	E
195	DWR-S4	10	2	AB	P
196	DWR-S5	3	1	AB	E
197	DWR-S6	2	1	BB	E
198	DWR-S7	3	1	AB	E
199	DWR-S8	16	1	AB	P
200	DWR-S9	3	1	AA	E

Specimen	Designation	# of Amplifi-cations	# of Sau Digests	Genotype	Estimate of DNA Quality
201	DWR-S10	7	1	BB	P
202	DWR-S11	3	1	AB	E
203	DWR-S12	7	1	BB	P
204	DWR-S13	7	1	AB	P
205	DWR-S14	7	1	AB	P
206	DWR-S15	6	1	BB	E
207	DKR-S1	6	2	AB	P
208	DKR-S2	7	2	BB	P
209	DKR-S3	2	0		P
210	DKR-S4	3	1	BB	P
211	DKR-S5	7	2	BB	P
212	DKR-S6	3	0		P
213	DKR-S7	2	0		P
214	DKR-S8	7	1	BB	P
215	DKR-S9	9	1	A_?	P
216	DKR-S10	9	1	AB	P
217	DKR-S11	10	1	BB	P
218	DKR-S12	2	1	AB	G
219	DKR-S13	5	1	BB	P
220	DKR-S14	7	0		P
221	DKR-S15	5	0		P
222	DKR-S16	12	1		P
223	DKR-S17	12	1		P
224	DKR-S18	11	2	BB	P
225	DKR-S19	12	1	BB	P
226	DKR-S20	12	1	BB	P
227	DKR-H1	8	0		P
228	DYR-P12	7	0		P

Table 2. Genotypes by locality of pallid, shovelnose, and hybrid sturgeons for a 1.7 kb prealbumin-related DNA sequence.

Locale	Shovelnose				Pallid				Hybrid				Lake				Locale Spelled Out
	AA	AB	BB	?	AA	AB	BB	?	AA	AB	BB	?	AA	AB	BB	?	
DYR**	0	3	17	0			11	1	-	-	-	-	-	-	-	-	Downstream of Yellowstone R. (Missouri R.)
DFP	0	5	15	0	0	0	1	0	-	-	-	-	-	-	-	-	Downstream of Pt. Peck (Missoouri R.)
UFP	0	3	16	1A-?	0	2	8	1	-	-	-	-	-	-	-	-	Upstream of Pt. Peck (Missouri R.)
DPOR	0	2	9	0	-	-	-	-	-	-	-	-	-	-	-	-	Downstream of Powder R. (Yellowstone R.)
DMR	-	-	-	-	0	2	1	0	1	5	13	1AB?	-	-	-	-	Downstream of Missouri R. (Mississippi R.)
BPFH	-	-	-	-	-	-	-	-	-	-	-	-	0	0	5	0	Blind Pony Fish Hatchery
DPR	-	-	-	-	0	0	2	0	-	-	-	-	-	-	-	-	Downstream of Platte R.
DOR	-	-	-	-	0	1	1	0	-	-	-	-	-	-	-	-	Downstream of Ohio R. (Mississippi R.)
ATR	2	6	12	0	0	4	6	0	0	5	5	0	-	-	-	-	Atchafalaya/Red River System
DO/GPNFH	-	-	-	-	0	0	5	0	-	-	-	-	-	-	-	-	Gavins Point NFH
YR	-	-	-	-	0	0	2	0	-	-	-	-	-	-	-	-	Downstream of Intake (Yellowstone R.)
WR***		10	3	1	-	-	-	-	-	-	-	-	-	-	-	-	White R.
DWR*	1	9	5	0	-	-	-	-	-	-	-	-	-	-	-	-	Downstream Wisconsin R.
DCD	0	1	0	0	-	-	-	-	-	-	-	-	-	-	-	-	Downstream of Cartersville Div./Yellowstone R.
DKR		3	9	7--? 1A-?	-	-	-	-				1--?	-	-	-	-	Missouri R. Downstream of Kansas R.

* Pure Shovelnose Population

** Pure Pallid Population

*** Speculated Spawning Population

Table 3.

Ratio of shared bands produced by PCR amplification with five single-copy nuclear gene primer pairs

Pairwise comparisons are above the diagonal. Proportion of bands shared with the total sturgeon data set are below the diagonal.

	Scaphirhynchus (Shovelnose) DFP-S8	*Scaphirhynchus* (Pallid) UFP-P1	*Acipenser fulvescens* BPL 1-5	*Acipenser oxyrhynchus* 135	*Acipenser oxyrhynchus* 224	*Acipenser oxyrhynchus* 353
Scaphirhynchus (Shovelnose) DFP-S8[a]	--	0.96	0.39	0.26	0.26	0.24
Scaphirhynchus (Pallid) UFP-P1[b]	0.55	--	0.38	0.28	0.29	0.27
Acipenser fulvescens[c] BPL 1-5	0.31	0.31	--	0.26	0.27	0.32
Acipenser oxyrhynchus 135[d]	0.20	0.22	0.20	--	0.85	0.84
Acipenser oxyrhynchus 224[e]	0.22	0.24	0.22	0.45	--	0.85
Acipenser oxyrhynchus 353[f]	0.20	0.22	0.24	0.43	0.47	--

Downstream of Fort Peck

Upstream of Fort Peck

Blind Pony Fish Hatchery

St. Lawrence River, Q

Pearl River, MS

Altamaha River, Georgia

ALABAMA STURGEON NUCLEAR DNA STUDY
SUMMARY, OCTOBER, 1994
GENETIC ANALYSES, INC.

The purpose of this report is to present results of a genetic study of tissues of a single Alabama sturgeon (labelled AS 94-7) compared with samples from two congeneric species, pallid and shovelnose sturgeons. The studies utilized methods and results of a larger study of genetic variability in pallid and shovelnose sturgeons throughout their geographic ranges, summarized in the document "*Scaphirhynchus* Final Report 10/94" prepared by Genetic Analyses.

GENETIC METHODS

Two types of genetic analyses of nuclear DNA were used in the present study, both based upon examination of DNA fragments generated using PCR (polymerase chain reaction) amplifications of sturgeon genomic DNA using specific DNA primers designed from highly conserved mammalian nuclear gene sequences. The first study determined the genotype of the Alabama sturgeon individual at a prealbumin-related locus demonstrated to be polymorphic in the earlier *Scaphirhynchus* work. Digestion of a 1.7 kilobase (kb) fragment amplified by PCR of sturgeon genomic DNA using prealbumin forward and reverse primers with Sau96I restriction endonuclease produced polymorphic restriction fragments falling into three phenotypes in agreement with Hardy-Weinberg expectations of two alleles at one locus: 1) 985 and 715 base pair (bp) fragments were designated as an *AA* genotype; 2) 375, 610, and 715 bp fragments (i.e., with a Sau96I cleavage site in the 985 bp fragment) were designated a *BB* genotype; and 3) 375, 610, 715, and 985 bp fragments were designated as an *AB* heterozygoous genotype. The second study provided a phenetic estimate of genetic relatedness of pallid, shovelnose, and Alabama sturgeons based upon the number of DNA bands shared in patterns produced by PCR amplification of sturgeon genomic DNA using forward and reverse primers for gastrin, prealbumin, and high mobility group protein-1 genes. In both studies, only bands reliably produced in 30 amplification cycles in three replicate amplifications were scored.

RESULTS AND DISCUSSION

1. Genotype at the prealbumin-related Sau96I fragment locus. The Alabama sturgeon studied clearly had the 375, 610, and 715 bp Sau96I fragments characteristic of the *BB* genotype. The *B* allele and *BB* genotype are the most common alleles and genotypes in pallid and shovelnose sturgeons, and also in lake and Atlantic *Acipenser* sturgeons.

2. Estimates of genetic identity of amplification fragments in Alabama, shovelnose, and pallid sturgeons using three PCR primers. The proportions of DNA fragments shared among the *Scaphirhynchus* sturgeon taxa are: pallid (downstream Yellowstone River-P7, prealbumin genotype BB) compared to

shovelnose (downstream Wisconsin River-S1 and S9, prealbumin genotypes AB and AA, respectively) sturgeons, 0.96; pallid compared to Alabama (AS94-7, prealbumin geonotype BB) sturgeon, 0.74; and shovelnose compared to Alabama sturgeon, 0.70. These results strongly suggest substantial genetic divergence of the Alabama sturgeon from both pallid and shovelnose congeners. The low proportion of shared amplimers was not the result of differentiation at a single locus: while gastrin-related sequences were less variable than prealbumin- and high mobility group protein-1-related sequences, each exhibited differences between the Alabama sturgeon and shovelnose and pallid samples. As an additional point of reference to genetic differentiation among sturgeon taxa in our previous study, the Alabama fragment-sharing average with other *Scaphirhynchus* taxa (~72%) falls between the average values for comparisons of *Acipenser fulvescens* and *A. oxyrhynchus* (~25%) and *A. o. oxyrhynchus* and *A. o. desotoi* (~85%). The Alabama sturgeon, then, based upon analysis of one individual, exhibits genetic differences from shovelnose and pallid sturgeons greater than those between described subspecies of an *Acipenser* taxon sampled from widely separated localities, but somewhat less than between two *Acipenser* species.

CONCLUSIONS

The data described here must be viewed as preliminary, but strongly indicate genetic differentiation of Alabama sturgeons from both pallid and shovelnose congeners. We should emphasize that many different pallid and shovelnose sturgeon DNA samples have been amplified by PCR with the three primers used for the Alabama sturgeon comparison, with the always reproducible result of virtually identical patterns of amplification fragments. While the Alabama sturgeon results are based upon DNA samples from a single individual, the reproducibly much greater differences in amplified fragments are striking. Should a second Alabama sturgeon individual yield mostly the same fragments upon amplification, the conclusion that much greater genetic differentiation exists between Alabama sturgeons and other *Scaphirhynchus* than between pallid and shovelnose sturgeons would be uncontestable. Expansion of sample sizes with regard to numbers of individuals and numbers of genetic loci (as well as, perhaps, comparisons of mitochondrial DNA sequences or restriction fragment patterns) should receive high priority to confirm the rather unexpectedly high estimate of genetic divergence of Alabama sturgeons. Because the Alabama sturgeon appears to be very uncommon, dedicated efforts to obtain tissue samples from a second individual should be undertaken as soon as possible.

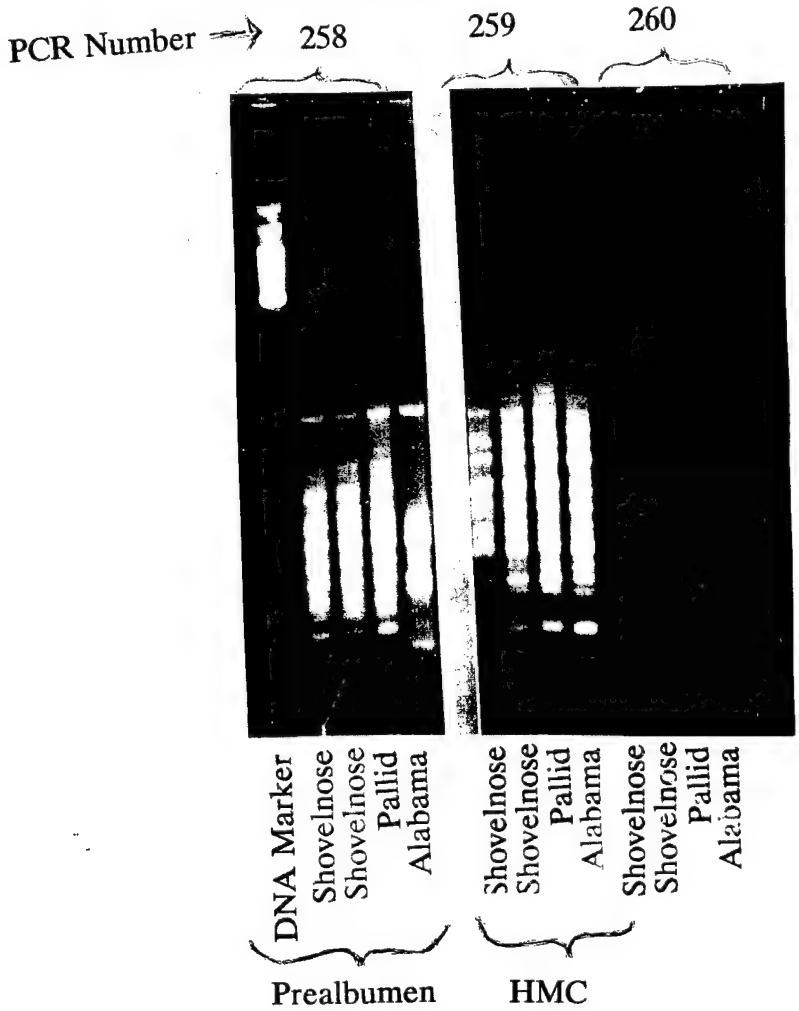
TABLE 4:

Ratio of shared bands produced by PCR amplification with three single-copy nuclear gene primer pairs

Pairwise comparisons are above the diagonal. Proportion of bands shared with the total sturgeon data set are below the diagonal.

	Shovelnose DWR-S1 (AB)& DWR-S9 (AA)	Pallid DYR-P7(BB)	Alabama AS94-7 (BB)
Shovelnose	----	0.96	0.70
Pallid	0.85	----	0.74
Alabama	0.70	0.74	----

DWR: Downstream Wisconsin River
DYR: Downstream of Yellowstone River (Missouri River)
(AB, AA, BB): genotypes determined using 1.7 kb PCR produc amplified using pre-albumin primers and restricte with Sau 96I



APPENDIX 1

SUMMARY OF CONTRACT FULFILLMENT
SCAPHIRHYNCHUS GENETIC TUDIES
GENETIC ANALYSES, INC.
6/94

ITEM 0001. Analysis of >200 tissue samples of *Scaphirhynchus* sturgeons was completed to estimate allele frequencies at a locus analyzed by presence or absence of polymorphic nuclear DNA fragments produced by digestion with Sau96I restriction endonuclease of a 1.7 kilobase fragment amplified by polymerase chain reaction (PCR) using forward and reverse primers for a prealbumin DNA sequence. No significant differences in allele frequencies between pallid and shovelnose sturgeon samples were detected, and the total data set was in close agreement with genotypic frequencies expected under Hardy-Weinberg equilibrium. A second data set to obtain estimates of genetic divergence using proportions of shared DNA fragments produced by PCR amplifications of five different primers likewise indicated minimal genetic divergence between pallid and shovelnose sturgeons. These results are in agreement with all other genetic studies of the taxa, and suggest that pallid and shovelnose sturgeons may in fact be conspecific.

ITEM 0002. No genetically meaningful management units (stocks) of pallid sturgeons could be detected, due to the genetic homogeneity described above. The absence of the prealbumin-related sequence *A* allele in areas producing morphologically apparently pure pallid sturgeons was noted, and the recommendation that no managed pallid stock should contain more than 10% *A* alleles was made as a precautionary compromise between genetic "purity" and loss of genetic variability.

ITEM 0003. The suggestion was made that the prealbumin-related sequence *A* allele may derive solely from shovelnose sturgeons, and that re-examination of apparent pallid individuals with *A* alleles should be made to reassess whether morphologic intermediacy could be detected. Documentation of allelic variability at only one locus precluded any assessment of introgression.

ITEM 0004. Homogeneity of allele frequencies at the prealbumin-related sequence locus among shovelnose sturgeon population samples suggested that sufficient gene flow exists to prevent formation of genetically meaningful management units (stocks). All available genetic data are concordant in failure to detect evidence of genetic differentiation.

SUMMARY OF ITEMS 0001 - 0004. The salient conclusion of all genetic studies conducted to date is that the specific status of pallid sturgeons needs to be critically reassessed. The possibility that the very slow rates of morphological evolution evident in sturgeons have parallels in rates of molecular evolution cannot be ignored, but the vastly greater genetic divergence observed in genetic analyses of *Acipenser* taxa support a conclusion that pallid and shovelnose sturgeons may be conspecific taxa.

APPENDIX 2

SCAPHIRHYNCHUS PROJECT

QUARTERLY PROGRESS REPORT OCTOBER, 1992

GENETIC ANALYSES, INC.

Techniques which do not require freezing were developed for stabilizing DNA in blood, muscle, and fin specimens collected in the field. Blood is withdrawn in EDTA and stored at room temperature in a lysis buffer which has been reported to maintain the integrity of DNA for years.

Total DNA was extracted from the first 117 blood specimens received (see appended). The DNA was quantitated by measuring its absorbance at 260 nm in a UV spectrophotometer, and each sample was checked on an agarose gel to determine its purity and to confirm the quantitation data (Fig. 1).

DNA was extracted using a modification of standard protocols including protease digestion, extraction with organic solvents, and ethanol precipitation. Modifications were necessary because most standard protocols are used for mammalian blood; because the presence of nuclei in fish erythrocytes drastically increases the quantity of DNA, much smaller volumes of blood are required. Initial extractions yielded huge brown gelatinous masses which proved impervious to all attempts to dissolve the material. After consultation with a colleague familiar with DNA extraction from reptiles, we reduced the volume of diluted blood from 7.5 ml to $\leq$0.6 ml and discovered that the material was much easier to handle and reproducibly yielded clean, soluble DNA.

DNA random primers that are currently available in groups of 20 for rapid scanning have been shown to have a shelf life of 4-6 weeks after solubilization. Consequently we waited until the majority of the specimens arrived before beginning the PCR analysis of the sturgeon blood DNA. A pilot experiment with two individuals (one shovelnose and one pallid) was conducted yielding no amplification (this is not to say that variation could not be detected with the use of many other primers). However, we are proceeding with an alternate PCR based method used by our collaborator, Dr. Jim Derr at Texas A&M University, which determines genetic variation without encountering some of the flaws inherent in RAPDs. To elucidate the advantages of this new technique, we here review both PCR based methods.

Recent advances in biotechnology and molecular biology now make it possible to quickly and economically assess genetic variation at the DNA level. The most significant accomplishment, which permits large-scale amplification of specific DNA sequences from very small (ng) starting material, is a technique termed Polymerase Chain Reaction (PCR). One early PCR based technique that population biologists utilize is called Randomly Amplified Polymorphic DNA

(RAPD). As the name implies, RAPDs are polymorphic DNA markers that are randomly amplified throughout an entire genome. The appeal of this technique is threefold. First, RAPD technology provides a rapid means for assessing genetic variation. In fact, with a given set of PCR primers, it is possible to complete an entire analysis for a number of individuals in a single day. Secondly, unlike some other techniques, i.e., DNA fingerprinting, the RAPD procedure does not require the use of radioactive materials and associated waste disposal problems. Finally, both theoretical and empirical results clearly demonstrate that RAPD markers are highly variable and potentially provide a high number of molecular genetic characters that can be used to address questions involving individuals, populations and species.

Nevertheless, research laboratories throughout the world have recently found that RAPD technology may not be the panacea originally claimed. One of the major disadvantages of this technique involves its inherent "randomness". Although RAPDs provide a method, apparently without bias, to determine levels of genetic variation throughout a genome, in practice the areas that are analyzed are anonymous DNA sequences. Therefore, although polymorphisms can be identified, it is extremely difficult to determine where these regions are in the genome and how they are related, for example, to structural genes. This, in turn, limits the inferences that can be made regarding these markers. In addition, the most powerful population genetic tests require one to identify a locus (=a gene) and different variants (=alleles) at that locus. Because RAPDs by default are random products it is seldom possible to determine what constitutes a "gene" and what is an "allele". Therefore, many important population parameters such as inbreeding, migration and population bottlenecks can not be determined with total confidence. Finally, the most serious drawback with RAPDs involves the reliability of these markers in subsequent analyses and among different researchers in various laboratories. Researchers at Texas A&M University and other major research institutions are reporting that assessment of these markers is extremely sensitive to very minute variations in the amount of DNA used in the reactions and the number of copies of the target sequence. In fact, this technique may be affected by levels of DNA that are even below the detection level of the most powerful measuring instruments currently available.

Due to advances in our ability to identify and utilize specific primer sequences, an alternate PCR based method for determining genetic variation recently has been developed. This technique has all of the advantages of RAPDs without the major disadvantages discussed above. Details of this procedure, termed Single Copy Gene Analysis, can be outlined as follows. First, isolate genomic DNA and perform PCR thermal cycles in the same fashion as with RAPD analysis except use specific primers that border a single gene

region. Secondly, determine the DNA sequence of this region and, finally, identify restriction enzymes that have a high probability of uncovering DNA sequence level variability in this gene. Following this "up front" information, population level data can then be produced by simply digesting the PCR products from a number of individuals with a suite of informative restriction enzymes and scoring genotypes directly from agarose gels.

The logic involved in the use of these single copy gene PCR primers is completely compatible with determining genetic variation and DNA markers from a number of individuals. The beauty of this technique is due to the fact that it is extremely powerful, very economical and conceptually quite simple. We are now entering an era where tools of what used to be considered "hard core molecular biology" can successfully be applied to questions involving non-human species. This technique in particular provides fast, accurate and, most importantly, unambiguous DNA level markers that are transportable and reproducible. Therefore, we propose to use this newly developed technique in the *Scaphirhynchus* study to yield superior and more informative results.

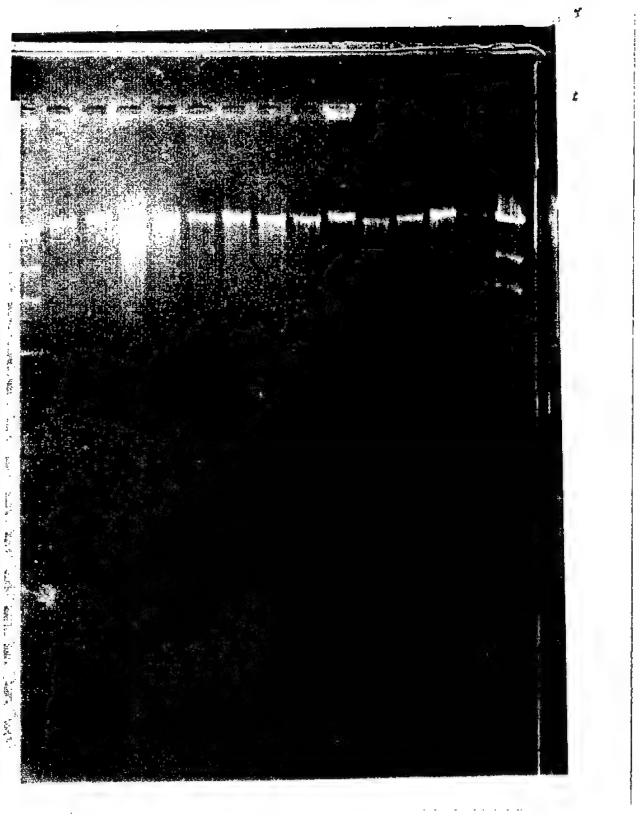


Figure 1. Ethidium bromide stained agarose gels of total DNA extracted from sturgeon blood. Lanes 1 and 15 are Lambda DNA markers digested with Hind III. Lanes 2-14 are sturgeon DNA representing 1% of the amount extracted (5 ul of 500 total). The sturgeon DNA is supercoiled and cannot be sized accurately with linear DNA markers, but its purity, estimated concentration, and relatively large size can be established by its staining pattern.

SCAPHIRHYNCHUS PROJECT

INTERIM PROGRESS REPORT FEBRUARY, 1993

GENETIC ANALYSES, INC.

Total DNA was extracted from 51 more blood specimens received. The DNA was quantitated by measuring its absorbance at 260 nm in a UV spectrophotometer, and each sample was checked on an agarose gel to determine its purity and to confirm the quantitation data.

DNA was extracted using a modification of standard protocols including protease digestion, extraction with organic solvents, and ethanol precipitation as explained in the October Progress Report. PCR (polymerase chain reaction) analysis of the sturgeon blood DNA was then begun using both shovelnose and pallid samples.

Twelve single copy nuclear genes (see appended) that have been conserved throughout many animal genomes were chosen. Through computer access to GenBank, any information available listing sequences of these particular genes in humans, rodents or bovine was accessed; a compare program was then run to align the sequences of different species. Coding regions (exons) and intervening sequences (introns) in these genes are known. Since exons code for proteins and are highly conserved to preserve the sequence and function of those proteins, they exhibit little polymorphism. Introns, however, tend to be highly polymorphic and lend themselves to genetic studies for this reason.

The gene sequences were analyzed with the intention of chosing primers that would be complementary to the conserved regions of exons that flank introns. After these regions were chosen, forward (5′ end to 3′ end) and reverse (3′ end to 5′ end) primers were designed based on parameters that are likely to produce good PCR primers, i.e., length of primer, melting temperature, and G/C content. Oligonucleotide primers were synthesized and PCR reactions were run with varying thermal profiles (temperature and times), magnesium concentrations, and genomic DNA concentrations. PCR products were analyzed on agarose gels.

We are currently investigating other single copy nuclear genes to design more primers. Next we will cut PCR products with restriction enzymes with short (4-6 base pairs) recognition sites and analyze their digest products on agarose gels to determine polymorphism between individuals. If our first battery of enzymes does not produce adequate data, one individual genomic DNA sample will be sequenced in the regions of the single copy nuclear genes of interest, and a computer analysis of the sequence will reveal a list of all known restriction enzymes that are likely to cut. PCR products will then be digested with this suite of enzymes and analyzed on agarose gels to acquire the maximum amount of data demonstrating polymorphism.

APPENDIX 1

Sturgeon genomic DNA was scanned for the following single copy nuclear gene PCR primers:

Locus		Primers
1 fatty acid binding protein 11-12 hundred bp (base pairs)		forward reverse
2 glucose-cerbose sidase 4-4.5 hundred bp		forward reverse
3 bovine adrenodoxin 700 bp		forward 1 forward 1A (internal -100 bp) reverse
4 bovine cytochrome c oxidase 700 bp		forward forward 2 forward 3 reverse
5 sterol co-desaturase 1800 bp		forward reverse
6 high mobility group protein I 1kb		forward reverse
7 thryothropin 650 bp		forward reverse
8 butytylcholinesterase	(9)	forward forward 1A reverse 2 reverse 2A

Locus	Primers
10	
prealbumin	forward
1200 bp	forward 1
	reverse
	reverse 2
11	
gastrin	forward
400 bp	reverse
12	
hydroxy ene steroid dehydrogenase	forward a
7-8 hundred bp	forward b
	forward c
	reverse d
	reverse e

All of these genomic DNAs will produce very good PCR products with the 16s rRNA conserved mitochondrial primers.

SCAPHIRHYNCHUS

INTERIUM REPORT 7/93

DNA was extracted from all of the blood samples received, bringing the total number of samples to 190. Previously described protocols were used.

Since pallids designated UFP and shovelnose sturgeons designated DPOR are likely to be the most genetically diverse populations, our PCR amplifications have focused on individuals from these two populations. Initial experiments using positive control (16S RNA) primer pairs were done to determine conditions that would amplify DNA from these templates. After these conditions were optimized, DNAs from several individuals from these two populations were amplified using a set of eight single copy nuclear gene primer pairs. These primers are listed in the following table:

Abbreviation	Gene Product
BAG	bovine adrenodoxin
COX	cytochrome c oxidase subunit V
GAST	gastrin
GLU	glucocerebrocidase
HMC	high mobility group protein I
PRE ALB	pre-albumin
THRY	thyrotropin
STERO	3beta-hydroxy-5-ene steroid dehydrogenase

These primers were designed based on consensus sequences of mammalian single copy nuclear genes available from GenBank, and the

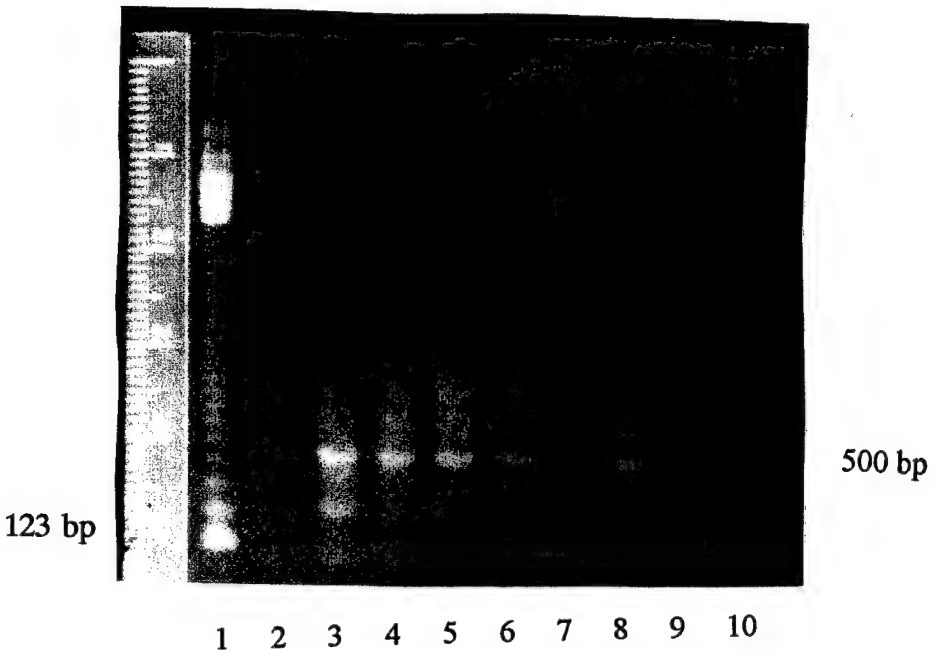
binding to fish genomic DNA results in amplification of more than one product. Numerous manipulations of reaction conditions have been shown to influence primer binding, and some of these have been used...showing mixed results.

The most obvious (and most promising) change is an increase in annealing temperature. Besides alterations in the thermal profile, there are also many additives to the reaction mixture that reportedly improve primer binding specificity and/or enzyme activity. The only one tried thus far is the addition of DMSO to the reaction which rarely alters the product. In the immediate future we will try adding formamide, NP-40, and tetramethyl-ammonium chloride. If a strong, single band is not consistently amplified using these variables, we will gel-purify the strongest band from the first PCR reaction and use those DNA, with the same primer pair, to re-amplify a product that is likely to be a single DNA fragment.

The next step will be restriction of that fragment, using the following enzymes (on page 3), which have short and/or frequently occurring recognition sequences to increase the probability of cutting.

RESTRICTION ENZYME	RECOGNITION SEQUENCE		RESTRICTION ENZYME	RECOGNITION SEQUENCE
Acc2	CG/CG		Mae1	C/TA/G
Aci1	C/CG/C		Mae2	A/CG/T
Alu1	AG/CT		Mae3	/GTNAC/
Apa1	G/GGCC/C		Mbo1	/GATC/
BamH1	G/GATC/C		Mse1	T/TA/A
Bcl1	T/GATC/A		Msp1	C/CG/G
BstU1	CG/CG		Mvn1	CG/CG
Cfo1	G/CG/C		Nla3	/CATG/
Dde1	C/TNA/G		Nla4	GGN/NCC
Dpn1	GA/TC		Pal1	GG/CC
Dpn2	/GATC/		Rma1	C/TA/G
Dra3	CAC/NNN/GTG		Rsa1	GT/AC
FnuD2	CG/CG		Sau3A1	/GATC/
Hae3	GG/CC		Sau96I	G/GNC/C
Hap2	C/CG/G		Spe1	A/CTAG/T
Hha1	G/CG/C		Taq1	T/CG/A
HinP1	G/CG/C			
Hpa1	GTT/AAC			
Hpa2	C/CG/G			

Note: Some of these restriction enzymes are isoschizomers (have the same recognition sequences), but they are included in this list because one of the forms frequently restricts more efficiently than the other(s) in a given system.



This gel shows PCR products of 4 pallids (UFP) and 4 shovelnose (DPOR) amplified using a GAST primer pair. The size of the predominant DNA fragment is small (~500 bp) but consistent. Lane 1 is a marker; lanes 2-5 are pallids; lanes 6-9 are shovelnose; lane 10 is the negative control, i.e., no template.

SCAPHIRHYNCHUS
INTERIM REPORT 1/94

The eight primer pairs listed in the 7/93 report have been used to amplify PCR products from several sturgeon DNA templates. The following variables have been adjusted in attempts to improve quantity of DNA amplified and to enhance specificity of primer binding (and consequently improve DNA quality):

- annealing temperatures
- primer:template ratios
- Mg^{+2} concentration
- re-amplification of aliquots of primary reaction
- re-amplification of gel-purified DNA from primary reaction
- addition to reaction mixture of:
  - DMSO
  - Tween 20
  - Tetramethyl ammonium chloride
  - Formamide
  - NP-40
  - Gelatin
  - BSA

After running over 1200 reactions and screening the eight primer pairs, seven primer pairs were found to yield either one small PCR product (300-800 bp) or too many products (so many bands that gel-purification resulted in contamination).

One primer pair, Pre-Albumin Forward and Reverse primers, produced 5-6 products during primary amplification with a low-stringency annealing temperature (52C). When these products were resolved and visualized in EtBr stained agarose, the brightest band comigrated with a 1.7 kb size marker, and the other bands were smaller than 1 kb (Figure 1). By sticking a needle in the 1.7 kb band and transferring a small amount of that band to a tube of H_2O, that product was gel-purified. After elution from the agarose, that DNA was used as template for PCR with a higher stringency annealing temperature (58C), producing a single band (1.7 kb) in 1% agarose (Figure 2).

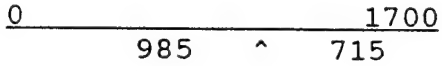
The DNA in the PCR reaction mix was used for endonuclease digestion without further purification. Several units of a restriction enzyme were incubated with the DNA in the specified buffer at the recommended temperature for 1-16 h. This reaction mix was electrophoresed in 3% agarose (2% regular:1% low-melting-temp.) and stained with EtBr. Molecular size markers and a fluorescent ruler were photographed with the samples to aid calculations of the apparent molecular length of the digest products.

Initially two specimens of shovelnose, two of pallid, and two hybrids were chosen based on the high yield and purity of product

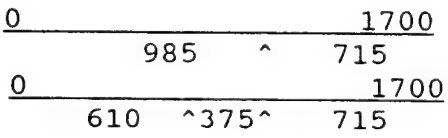
amplified with Pre-albumin primers. These six specimens were used to screen thirty-one restriction enzymes, of which fourteen have 4-bp recognition sequences, 16 have 6-bp recognition sequences, and one has an 8-bp recognition sequence. Eleven of the 4-base cutters and eight of the 6-base cutters digested the 1.7 kb DNA. Only one enzyme, Sau 96I, showed a variant restriction pattern among the six specimens initially screened (Table 2). Figure 3 shows a representative gel of six DNAs that were cut by a restriction enzyme to produce identical patterns in each specimen. Figure 4 shows a gel of six DNAs that were incubated with a restriction enzyme that failed to cut. Figure 5 shows a gel of six DNAs cut with Sau 96I, showing variation.

Subsequently six more shovelnose, eight more pallid, two more hybrids, and five lake sturgeon DNAs were amplified and restricted. The following model is proposed and the data analyzed thus far are summarized in Table 1. All sizes are approximate.

HOMOZYGOUS-1 SITE (*AA*)



HETEROZYGOUS (*AB*)



HOMOZYGOUS-2 SITES (*BB*)

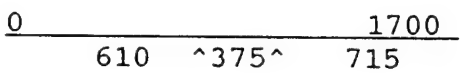


Table 1.

SHOVELNOSE		PALLID		HYBRID		LAKE	
192(DWR-S1)	*AB*	74 (DYR-P1)	*BB*	82(DMR-H6)	*BB*	97(BPL-1)	*BB*
193(DWR-S2)	*AB*	109(DYR-P2)	*BB*	83(DMR-H7)	*AB*	98(BPL-2)	*BB*
194(DWR-S3)	*BB*	110(DYR-P3)	*BB*	90(DMR-H14)	*BB*	99(BPL-3)	*BB*
196(DWR-S5)	*AB*	112(DYR-P5)	*BB*	91(DMR-H15)	*AB*	100(BPL-4)	*BB*
197(DWR-S6)	*BB*	113(DYR-P6)	*BB*			101(BPL-5)	*BB*
198(DWR-S7)	*AB*	114(DYR-P7)	*BB*				
200(DWR-S9)	*AA*	103(DPR-P2)	*BB*				
202(DWR-S11)	*AB*	104(DOR-P1)	*BB*				
		106(DMR-P1a)	*AB*				
		107(DMR-P1b)	*BB*				
RATIOS SUMMARIZED							
2 *BB* : 5 *AB* : 1 *AA*		9 *BB* : 1 *AB* : 0 *AA*		2 *BB* : 2 *AB* : 0 *AA*		5 *BB* : 0 *AB* : 0 *AA*	

Assuming Hardy-Weinberg equilibrium, the above model would predict the following allelic frequencies for the eight shovelnose described above:

Of 16 alleles, 7 are *A* (7/16=0.44) and 9 are B (9/16=0.56). If $p^2:2pq:q^2$, then the frequency of *AA* would be $(0.44)^2$ or 0.19, the frequency of *BB* would be $)0.56)^2$ and the frequency of *AB* would be 2(0.56 X 0.44) or 0.49. The expected number of individuals would be 1.53 *AA* : 3.94 *BB* : 2.51 *BB*. If expected ratios are compared with the observed ratios, a chi-square test would give a probability of 0.57.

Currently these studies are being continued and expanded. Because DNA from *Acipenser fulvescens* produced a PRC product identical in size and with a restriction pattern similar to that of *Scaphirhynchus*, we have requested DNAs from three *A. oxyrhynchus oxyrhynchus* and one *A. oxyrhynchus desotoi*. Amplification of the same PCR product and analysis of those restriction digests will allow us to assess in a related genus the expected levels of specific and intra-specific geographic genetic variation. It may be significant to note that in *A. fulvescens* the *B* allelle was the only one present, suggesting that it may be the most primitive allele for this gene in sturgeon species.

With these data for comparison, we will complete analysis of shovelnose and pallids to compare intraspecific geographic variation and potential interspecific allelic frequency variation.

Table 1. Summary of restriction digests of 1.7 kb PCR product amplified by Pre-Albumin primer pair

RESTRICTION ENZYME	RECOGNITION SEQUENCE	SHOVELNOSE	PALLID	HYBRID	CUT?	POLYMORPHIC ?
AatII	GACGT/C	192,193	113,114	82,83	+	-
AciI	C/CGC	192,192	113,114	82,83	+	-
BamHI	G/GATCC	192,193	113,114	82,83	+	-
BbsI	GAAGACN2/	192,193	113,114	82,83	-	-
BfaI	C/TAG	192,193	113,114	82,83	+	-
BsiWI	C/GTACG	192,193	113,114	-	+	-
BssHII	G/CGCGC	192,193	113,114	82,83	-	-
BstUI	CG/CG	192,193	113,114	82,83	+	-
Bsu36I	CC/TNAGG	192,193	113,114	-	-	-
DdeI	C/TNAG	192,193	113,114	82,83	-	-
DpnI	GA/TC	192,193	113,114	82,83	-	-
DpnII	/GATC	192,193	113,114	82,83	+	-
DraI	TTT/AAA	192,193	113,114	82,83	-	-
EcoRI	G/AATTC	192,193	113,114	82,83	+	-
HaeIII	GG/CC	192,193	113,114	82,83	+	-
HhaI	GCG/C	192,193	113,114	82,83	+	-
HincII	GTPy/PuAC	192,193	113,114	82,83	+	-
HindIII	A/AGCTT	192,193	113,114	82,83	+	-
HinfI	G/ANTC	192,193	113,114	82,83	+	-
HpaI	GTT/AAC	192,193	113,114	82,83	-	-
MspI	C/CGG	192,193	113,114	82,83	-	-
NlaIII	CATG/	192,193	113,114	82,83	+	-
NotI	GC/GGCCGC	192,193	113,114	82,83	-	-
RsaI	GT/AC	192,193	113,114	82,83	+	-
SacI	GAGCT/C	192,193	113,114	82,83	+	-
Sau96I	G/GNCC	192,193,194,196, 197,198,200,202	74,103,104,106,107,109,110,112,113,114	82,83,90,91	+	+
ScaI	AGT/ACT	192,193	113,114	82,83	+	-
SmaI	CCC/GGG	192,193	113,114	82,83	+	-
StuI	AGG/CCT	192,193	113,114	82,83	-	-
TaqI	T/CGA	192,193	113,114	82,83	+	-
XhoI	C/TCGAG	192,193	113,114	-	-	-

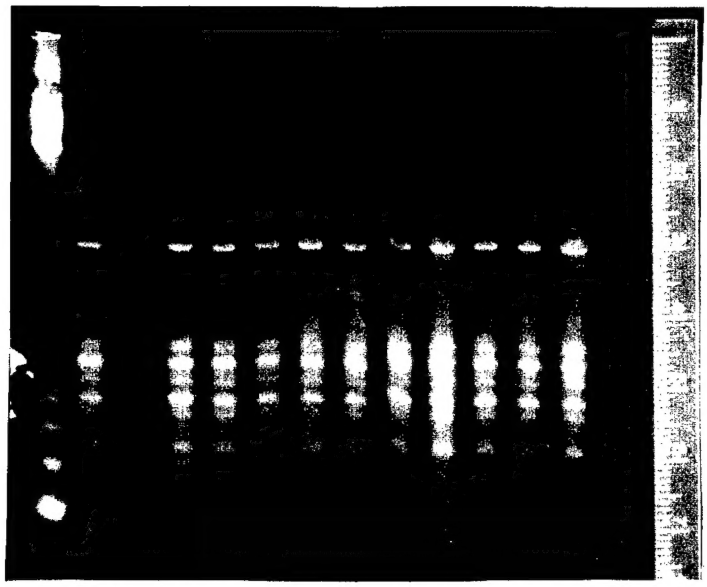


Figure 1. PCR products after primary amplification of genomic DNAs from four shovelnose, four pallids, and four hybrid sturgeons. Lane 1, BRL 123 bp DNA ladder. Lanes 2-5, ST 194-197 (shovelnose). Lanes 6-9, ST 74, 109-111 (pallid). Lanes 10-13, ST 77-80 (hybrids).

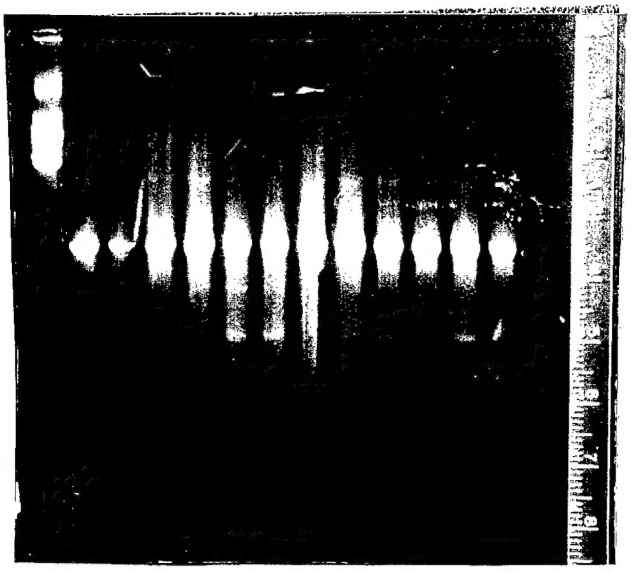


Figure 2. Secondary amplification of 1.7 kb gel-purified primary PCR product. Lane 1, BRL 123 bp DNA ladder. Lanes 2-5, ST 192-3 (shovelnose). Lanes 6-9, ST 113-4 (pallid). Lanes 10-13, ST 82-3 (hybrid sturgeon).

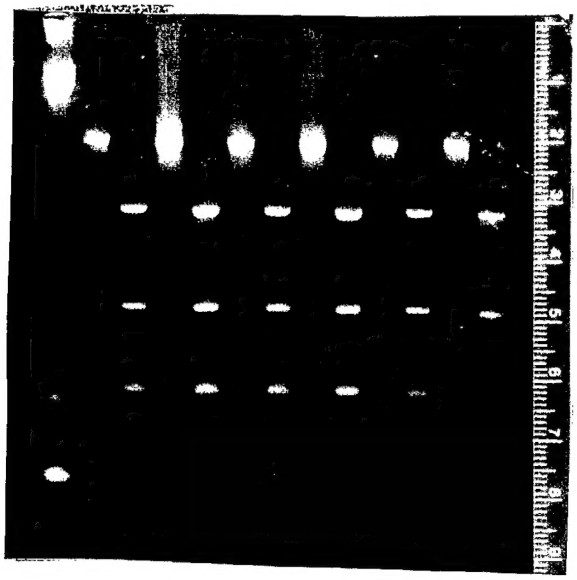


Figure 3. 1.7 kb PCR product from six individuals after digestion with a restriction enzyme, Rsa I. Lane 1, 123 bp DNA ladder. Lanes 2, 4, 6, 8, 10, and 12: DNA incubated with buffer and no enzyme. Lanes 3, 5, 7, 9, 11, and 13: DNA incubated with Rsa I. Specimens are ST 192, 193, 113, 114, 82, and 83. Note that the digest products appear identical in the six individuals.

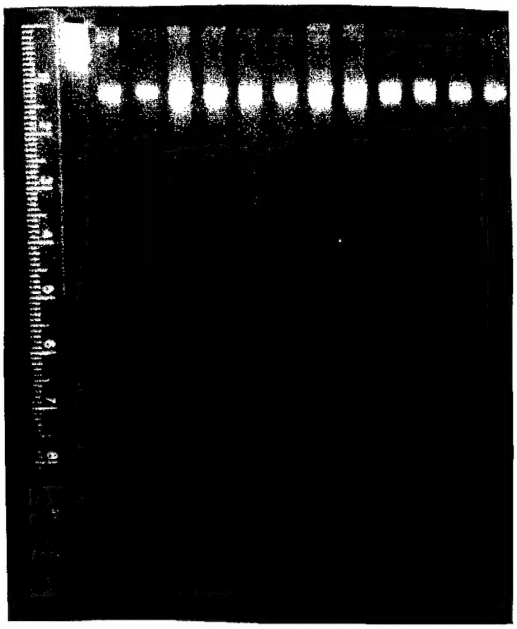


Figure 4. 1.7 kb PCR product from six individuals after digestion with a restriction enzyme, Stu I. Lane 1, 123 bp DNA ladder. Lanes 2, 4, 6, 8, 10, and 12: DNA incubated with buffer and no enzyme. Lanes 3, 5, 7, 9, 11, and 13: DNA incubated with Stu I. Specimens are ST 192, 193, 113, 114, 82, and 83. Note that the digest products appear identical in the six individuals.

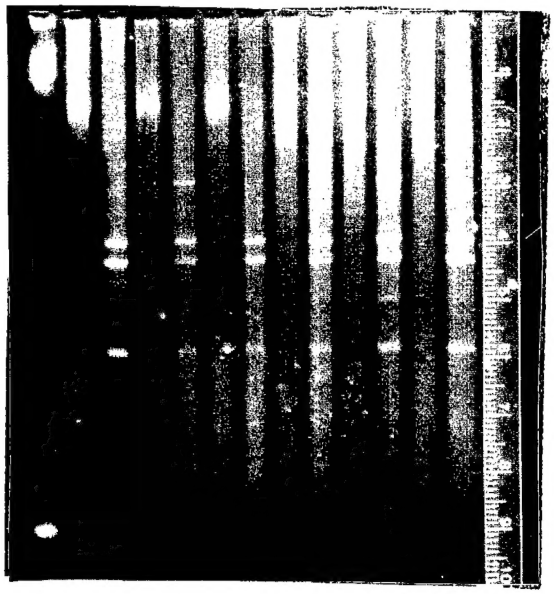


Figure 5. 1.7 kb PCR product from six individuals after digestion with a restriction enzyme, Sau 96I. Lane 1, 123 bp DNA ladder. Lanes 2, 4, 6, 8, 10, and 12: DNA incubated with buffer and no enzyme. Lanes 3, 5, 7, 9, 11, and 13: DNA incubated with Sau 96I. Specimens are ST 192, 193, 113, 114, 82, and 83. Note that the digest products do not appear identical in the six individuals. According to the model below, Lanes 3, 7, 9 and 13 show a *BB* genotype, and Lanes 5 and 11 show an *AB* genotype.

HOMOZYGOUS-1 SITE (*AA*)

0 1700
985 ^ 715

HETEROZYGOUS (*AB*)

0 1700
985 ^ 715

0 1700
610 ^375^ 715

HOMOZYGOUS-2 SITES (*BB*)

0 1700
610 ^375^ 715

Predicted DNA bands in an agarose gel:

Enzyme:	-	+	-	+	-	+
1700>	___		___		___	
985>		___		___		
715>		___		___		
610>				___		___
375>				___		___